embB 306 Mutations as Molecular Indicators to Predict Ethambutol Susceptibility in Mycobacterium tuberculosis

Sirgel, Frederick A ; Warren, Robin M ; Streicher, Elizabeth M ; Victor, Thomas C ; van Helden, Paul D ; Böttger, Erik C

Abstract: Background: Discordant results in conventional susceptibility testing of ethambutol against Mycobacterium tuberculosis may lead to underreporting of drug resistance. Methods: A 240-bp region of the embB gene in 111 clinical isolates of M. tuberculosis was sequenced and examined for mutations linked to ethambutol resistance. The phenotypic susceptibility levels of the isolates were quantified by the BACTEC™ MGIT 960™ TB System and correlated with the genotypic test results. These data were analyzed to find information that could be used to clarify discordant ethambutol susceptibility test results. Results: Mutations M306I (n = 56), M306V (n = 18) and M306L (n = 3) in M. tuberculosis showed decreased susceptibility to ethambutol. The minimum inhibitory concentrations (MICs) in 73% (56/77) of embB306 mutants were at or just above the critical concentration (MICs, 5.0 to 12.5 µg/ml) of ethambutol reflecting borderline (or intermediate) resistance. Eight ethambutol-resistant isolates lacked embB mutations, probably due to mutational alterations elsewhere in the genome. Conclusion: Our findings suggest that clinical isolates containing embB306 mutations with MICs overlapping the critical concentration are associated with discordant ethambutol susceptibility test results. The clinical significance of borderline resistance in combination treatment of tuberculosis remains to be determined before alternative ethambutol breakpoints are considered.

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*embB*306 Mutations as Molecular Indicators to Predict Ethambutol Susceptibility in *Mycobacterium tuberculosis*

Frederick A. Sirgel[a] Robin M. Warren[a] Elizabeth M. Streicher[a]
Thomas C. Victor[a] Paul D. van Helden[a] Erik C. Böttger[b, c]

[a]DST/NRF Centre of Excellence for Biomedical TB Research/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Faculty of Health Science, Stellenbosch University, Cape Town, South Africa; [b]Institut für Medizinische Mikrobiologie and [c]Nationales Zentrum für Mykobakterien, Universität Zürich, Zürich, Switzerland

Key Words
Antituberculosis drugs · Borderline resistance · Tuberculosis therapy · Mutations · Drug resistance

Abstract
Background: Discordant results in conventional susceptibility testing of ethambutol against *Mycobacterium tuberculosis* may lead to underreporting of drug resistance. ***Methods:*** A 240-bp region of the *embB* gene in 111 clinical isolates of *M. tuberculosis* was sequenced and examined for mutations linked to ethambutol resistance. The phenotypic susceptibility levels of the isolates were quantified by the BACTEC™ MGIT 960™ TB System and correlated with the genotypic test results. These data were analyzed to find information that could be used to clarify discordant ethambutol susceptibility test results. ***Results:*** Mutations M306I (n = 56), M306V (n = 18) and M306L (n = 3) in *M. tuberculosis* showed decreased susceptibility to ethambutol. The minimum inhibitory concentrations (MICs) in 73% (56/77) of *embB*306 mutants were at or just above the critical concentration (MICs, 5.0 to ≤12.5 μg/ml) of ethambutol reflecting borderline (or intermediate) resistance. Eight ethambutol-resistant isolates lacked *embB* mutations, probably due to mutational alterations elsewhere in the genome. ***Conclusion:*** Our findings suggest that clinical isolates containing *embB*306 mutations with MICs overlapping the critical concentration are associated with discordant ethambutol susceptibility test results. The clinical significance of borderline resistance in combination treatment of tuberculosis remains to be determined before alternative ethambutol breakpoints are considered.

Introduction

Ethambutol (EMB) inhibits arabinogalactan synthesis and resistance to EMB is associated with mutations in three genes involved in arabinogalactan synthesis termed *emb*CAB [1]. These genes are organized as a 10-kbp operon, designated the EMB-resistance-determining region [1]. The most frequent point mutations associated with EMB resistance occur at codon 306 in the *embB* gene (*embB*306) [1–3]. Single nucleotide polymorphisms (SNPs) described in this codon are ATG → GTG, CTG, ATA, ATC and ATT, resulting in three amino acid substitutions methionine (M) (ATG) → valine (V) (GTG), leucine (L) (CTG) and isoleucine (I) (ATA; ATC; ATT) [1, 3]. Allelic exchange experiments demonstrated that mutations at *emb*B306 are associated with decreased sus-

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Dr. F.A. Sirgel
Division of Molecular Biology and Human Genetics
Faculty of Health Science, Stellenbosch University
PO Box 19063, Cape Town 7505 (South Africa)
E-Mail fas@sun.ac.za

ceptibility to EMB [2–4]. Sequence variation in *embB*306 has therefore been proposed as a promising molecular marker to predict EMB resistance in *Mycobacterium tuberculosis* [2]. However, reports that mutations in *embB*306 were also present in EMB-susceptible strains question the reliability of this mutation as a reliable marker of resistance [5, 6].

Disparity in phenotypic susceptibility results of EMB has widely been reported and various reasons have been linked to poor reproducibility [1, 7, 8]. Among these are methodological variations, heteroresistance, and the likelihood that the narrow range between the minimum inhibitory concentrations (MICs) of wild-type and resistant isolates give rise to borderline results [2, 7–9].

The aim of this study was (1) to correlate mutations in the *embB* gene at codon 306 with the MIC levels of EMB in clinical isolates of *M. tuberculosis* and (2) to analyze the quantitative resistance and molecular data to clarify inconsistencies in routine EMB susceptibility test results.

Materials and Methods

A total of 111 drug-resistant *M. tuberculosis* clinical isolates were selected for study. The isolates were obtained from separate patients resident in the Western Cape, South Africa. Aliquots of stock cultures were prepared and kept under cryogenic conditions (–80°C). The resistance status of all isolates was confirmed by BACTEC™ 960™ testing (Becton Dickinson Diagnostic Systems, Sparks, Md., USA) according to the standard critical concentrations set by the World Health Organization [10]. Fifty-two isolates were extensively drug-resistant (XDR), 47 were multidrug-resistant (MDR), 10 were resistant to both isoniazid (INH) plus streptomycin and two were resistant to INH. The genotypes of all selected isolates were previously determined and categorized as typical Beijing (n = 31), atypical Beijing (n = 49) [11] and Low Copy Clade (LCC; n = 31) strains [12]. Within the typical Beijing group were 8 XDR and 23 MDR strains; the atypical Beijing family was comprised of 30 XDR, 17 MDR and 2 INH monoresistant strains. The LCC included 14 XDR, 7 MDR and 10 isolates that were resistant to INH and streptomycin.

All the isolates were screened for SNPs in a 240-bp region of the *embB* gene, which encodes amino acids 271–350. This region was amplified by PCR with primer sets as previously described [13]: Emb151 (5′-CGGCATGCGCCGGCTGATTC-′3) and EmbB131 (5′-TCCACAGACTGGCGTCGCTG-′3). Amplicons were sequenced with an ABI PRISM DNA sequencer (Applied Biosystems, Foster City, Calif., USA) and the resulting chromatograms were analyzed by the use of Chromas software (Technelysium Pty Ltd).

The MICs for EMB were determined by quantitative drug susceptibility testing in BACTEC MGIT 960 eXtended individual Susceptibility Testing (TB eXiST) for EpiCenter™ V5.75A (BD Bioscience, Erembodegem, Belgium) as described [14]. EMB (Sigma-Aldrich, Kempton Park, South Africa) was prepared in sterile distilled water, filter-sterilized and stored at –80°C for up to 6 months. All isolates were subjected to quantitative drug susceptibility testing at EMB concentrations of 1.25, 2.5, 5.0, 12.5, 25.0 and 50.0 μg/ml. The interpretation of MIC values was based on the 1% proportional method. A critical concentration of 5.0 μg/ml was used to distinguish between EMB-susceptible and EMB-resistant isolates [10]. *M. tuberculosis* H37Rv (ATCC 27294) was included as a drug-susceptible reference strain for quality control purposes.

Results

The distribution of *embB*306 mutations amongst 111 clinical isolates of three different genotypes, together with sequence and quantitative drug susceptibility data, is presented in tables 1 and 2. Thirty-four (31%) of the 111 isolates lacked *embB*306 mutations and 76% (26/34) of these exhibited EMB MICs of 1.25 to ≤2.5 μg/ml, which are below the standard critical concentration of 5.0 μg/ml [10] (tables 1, 2). The susceptibility levels of these isolates were similar to those displayed by the susceptible *M. tuberculosis* reference strain (H37Rv) confirming that they are genotypically and phenotypically susceptible to EMB. The 26 isolates were identified to be of the typical Beijing (n = 6), atypical Beijing (n = 4) and LCC (n = 16) genotypes. Amongst the 26 isolates were 2 XDR, 12 MDR, 2 INH monoresistant and 10 INH plus streptomycin-resistant strains. Eight of the 34 isolates that had no *embB* mutations showed phenotypic resistance to EMB exhibiting MICs of >5.0 to ≤12.5 μg/ml (n = 1), >12.5 to ≤25.0 μg/ml (n = 6) and >25.0 to ≤50.0 μg/ml (n = 1). All 8 isolates were typical Beijing strains and 4 were MDR and 4 XDR.

In comparison, 69% (77/111) of the test isolates had SNPs at *embB*306 with 4 different nucleotide substitutions (ATG→ATC, ATA, GTG and CTG) associated with decreased susceptibility to EMB. M306I substitutions occurred as a result of nucleotide alterations at ATG→ATC (n = 32) and ATG→ATA (n = 24) in 56/77 (73%) isolates. Eighteen (23%) of the 77 isolates contained an M306V (ATG→GTG) mutation and 3/77 (4%) had an M306L (ATG→CTG) replacement (table 1). Among the 77 mutant isolates, 67 (87%) were phenotypic resistant and 10 (13%) phenotypic susceptible to EMB as per critical concentration testing [10]. The EMB MIC of the 10 isolates was equivalent to the critical concentration (5.0 μg/ml) and 2- to 4-fold higher than those of the 26 susceptible wild-type isolates. Within the typical Beijing family 55% (17/31) had *embB*306 mutations, dominated by M306V

Table 1. Correlation of phenotypic and genotypic susceptibility data of EMB in different *M. tuberculosis* genotypes

Genotypes	MIC μg/ml	Isolates with *emb*B306 mutations (n = 77)				Wild-type *embB*306 isolates
		M306I		M306V	M306L	
		ATG→ATC	ATG→ATA	ATG→GTG	ATG→CTG	
Typical Beijing (n = 31)	1.25 to ≤2.5	0	0	0	0	6
	5.0	0	0	0	0	0
	>5 to ≤12.5	0	1	1	0	1
	>12.5 to ≤25	1	0	13	0	6
	>25 to ≤50	0	0	1	0	1
Atypical Beijing (n = 49)	1.25 to ≤2.5	0	0	0	0	4
	5.0	1	1	0	0	0
	>5.0 to ≤12.5	19	17	1	0	0
	>12.5 to ≤25	3	0	0	3	0
	>25 to ≤50	0	0	0	0	0
LCC (n = 31)	1.25 to ≤2.5	0	0	0	0	16
	5.0	5	2	1	0	0
	>5.0 to ≤12.5	3	3	1	0	0
	>12.5 to ≤25	0	0	0	0	0
	>25 to ≤50	0	0	0	0	0
n		32	24	18	3	34

Table 2. Summary of EMB MICs compared to *embB*306 mutations detected in *M. tuberculosis* isolates

MIC μg/ml	Isolates with *embB*306 mutations (n = 77)				Wild-type *embB*306 isolates
	M306I		M306V	M306L	
	ATG→ATC	ATG→ATA	ATG→GTG	ATG→CTG	
1.25 to ≤2.5	0	0	0	0	26
5.0	6	3	1	0	0
>5.0 to ≤12.5	22	21	3	0	1
>12.5 to ≤25	4	0	13	3	6
>25 to ≤50	0	0	1	0	1
n	32	24	18	3	34

(88%; 15/17). Thirteen (87%; 13/15) M306V mutants had EMB MICs of >12.5 to ≤25.0 μg/ml and 2/15 exhibited MICs of >5.0 to ≤12.5 μg/ml and >25 to ≤50 μg/ml, respectively. Only 2/17 M306I mutations were detected among the typical Beijing isolates; one of them had an MIC of >5.0 to ≤12.5 μg/ml and the other one showed an MIC of >12.5 to ≤25.0 μg/ml. Among the atypical Beijing isolates, 92% (45/49) had SNPs in *embB*306 and of these 91% (41/45) possessed the M306I mutations, ATC (n = 23) and ATA (n = 18). The EMB MICs in 38/41 (93%) of these isolates were 5.0 to ≤12.5 μg/ml reflecting low-level or intermediate resistance. Higher MICs (>12.5 to ≤25.0 μg/ml) were demonstrated in only 3/41 (7%) of the atypical Beijing isolates with the M306I substitution (ATG→ATC). The other four isolates within this genotype had M306V (n = 1) and M306L (n = 3) mutations with EMB MICs of >5.0 to ≤12.5 and >12.5 to ≤25 μg/ml, respectively. *embB*306 mutations were detected in 48% (15/31) of the LCC isolates and 87% (13/15) of these harbored the M306I mutation, while the amino acid substitution M306V was present in 2/13 of the isolates. All 15 LCC mutant isolates exhibited EMB MICs (5.0 to ≤12.5) similar to those observed in most of the atypical Beijing strains.

Discussion

Amongst the 56 isolates with M306I substitutions, 93% (52/56) had MICs equal or moderately above the critical concentration (MICs, 5.0 to ≤12.5 μg/ml). We suggest that these borderline results account for the discordance found among isolates that were genotypic resistant but phenotypic susceptible. This problem can be reduced by adjusting the current critical concentration or by introducing two or multiple breakpoints, based on measures of quantitative drug susceptibility and genotypic testing. A susceptibility breakpoint of 4.0 μg/ml for EMB has recently been suggested [15]. This recommendation was based on a pharmacokinetic (PK) and pharmacodynamic (PD) approach, but it failed to specify the relevant in vitro conditions for susceptibility testing for which this breakpoint is suitable [15]. Using a reduced breakpoint to detect phenotypic resistance should improve the detection rate of mutants that confer low-level resistance to EMB. It is, however, important to introduce an assessment scheme that identifies intermediate susceptible strains, based on the MIC distribution of EMB. Patients with moderately increased MICs may still benefit from treatment, especially in situations where therapeutic options are limited, since low-level (borderline) resistance does not necessarily imply clinical resistance.

EMB has a rather narrow therapeutic index as a result of ocular toxicity which is dose-related [16]. The standard recommended daily doses of EMB for adults are 15–20 mg/kg body weight daily, or 3 times weekly at 25–35 mg/kg body weight [16]. A peak serum level of 5.0 μg/ml is achieved following a dose of 25 mg/kg of EMB [16], which is equivalent to the MICs of low-level (or borderline) EMB-resistant isolates. EMB serum concentrations are dose related and 10 μg/ml can be attained at a dose of 50 mg/kg, but with an increased risk of patients developing ocular toxicity [16]. The relation between PK and PD parameters (PK/PD index) is considered an important characteristic of drug efficacy [15]. The PK/PD index for EMB according to the above-mentioned C_{max}/MIC data reflects low therapeutic efficacy. Despite this, EMB remains effective even at sub-MICs, as it has a synergistic effect when used with companion drugs [10]. A thorough clinical assessment involving appropriate PK/PD parameters needs to be done to measure the clinical impact of decreased EMB susceptibility in particular on MDR and XDR tuberculosis (TB).

Using MIC values as a microbiological parameter can be misleading, since a critical concentration of 5.0 μg/ml in MGIT 960 for EMB is equivalent to 2.0, 2.5, 5.0 and 7.5 μg/ml in Löwenstein-Jensen, BACTEC 460, 7H10 and 7H11 media, respectively [4, 10]. Previous studies demonstrated that susceptibility testing of EMB on agar-based medium can lead to underreporting of 50–91% of drug resistance compared with genotypic and radiometric BACTEC 460 testing [2, 8, 9, 13]. Borderline resistance to EMB in clinical isolates was successfully detected in this study by quantitative drug susceptibility testing using MGIT 960 equipped with TB eXiST [14]. This technology is thus recommended to become the standard for susceptibility testing of EMB, given that agar-based critical methods are unreliable.

Fourteen (78%) of 18 isolates with the M306V mutation had MICs >12,5 μg/ml, which are well above the critical concentration. Only one isolate with this mutation was phenotypic susceptible to EMB with an MIC of 5.0 μg/ml (borderline result), while three had moderate levels of resistance (MICs; >5.0 to ≤12.5 μg/ml). The M306L mutation was detected in only three isolates with resistance levels similar to those observed in the majority of the M306V mutant isolates (MICs; >12.5 to ≤25.0 μg/ml). M306V and M306L were thus associated with higher levels of EMB resistance as opposed to those generated by the M306I substitution. Similar observations were made in previous studies [1–3, 17, 18], which also implied that M306L is less frequently found in *M. tuberculosis* strains compared to M306I and M306V. Isolates that acquired either the M306I or M306V mutations had variable EMB MICs within a concentration range of 5.0–50.0 μg/ml. However, a fairly high proportion of isolates 43/56 (77%) with the M306I mutation had MICs clustered in a narrow interval (>5 to ≤12.5 μg/ml) adjacent to the critical concentration of 5.0 μg/ml. This interval was flanked by 16% (9/56) of the isolates with MICs equivalent to the critical concentration, while 7% (4/56) displayed higher levels of resistance (>12.5 to ≤25 μg/ml). Similarly, 72% (13/18) of the M306V mutants had MICs of >12.5 to ≤25 μg/ml, while 22% (4/13) were below and 6% (1/18) above these concentrations. These data illustrate that the MIC distributions for EMB followed a typical gaussian pattern [7], suggesting that the majority of the isolates possessed a single mechanism of resistance. The co-existence of other point mutations that were not investigated in this study could be the reason for higher levels of resistance. However, it has previously been shown that single *embB* mutations are responsible for low to moderate levels of EMB resistance [1, 3, 17, 19]. Based on this assumption, it has been anticipated that two or more independent mutations are needed to mediate high-level EMB resistance in *M. tuberculosis* [19]. In

this study, a high proportion (56/77; 73%) of *embB* mutants had low to moderate levels of EMB resistance, which is additional evidence that single point mutations are the sole causative reason for resistance. The isolates with MICs above 12.5 μg/ml were mainly associated with the M306V and M306L mutations, which are known to mediate higher levels of resistance [1–3, 18]. Only one of these isolates had an MIC 5-fold higher than the critical concentration. Additional resistance mechanisms or mutations could have been involved [17, 19] in this case, but this study lacks information to confirm or exclude this possibility. Based on the above hypothesis and published evidence [2, 3, 19], it is unlikely that secondary mutations influenced the susceptibility levels of most of the clinical isolates that were quantified in this study. Decreased susceptibility in 8 isolates that lacked *embB*306 mutations points to the presence of sequence alterations elsewhere in the 10-kbp *emb*CAB operon or in other regions of the genome [1–4, 17–22]. Alternative mechanisms or efflux [23], rather than genetic changes, may also contribute to decreased EMB susceptibility [4, 17, 19, 20]. The mechanisms responsible for EMB resistance are diverse and complex [4, 17–23] and need further investigation to clarify the actual molecular basis of EMB resistance.

M306I was the most frequently observed mutation (56/77) in this study, followed by M306V (18/77) and then M306L (3/77). M306I was more prevalent in the atypical Beijing (41/77) and LCC (13/77) genotypes and was mainly correlated with a lower level of EMB resistance (5 to ≤12.5 μg/ml). M306V were linked to typical Beijing isolates, while M306L was relatively rare and found in 3 atypical Beijing strains. The Beijing genotype has been associated with younger patients, which reveals a high transmission rate amongst individuals in this age group [24]. Beijing isolates are also frequently associated with MDR TB and serious efforts should therefore be made to prevent its transmission [24].

Conclusion

Phenotypic and genotypic analyses demonstrated that *embB*306 mutations were reliable indicators of EMB susceptibility in a substantial proportion of 111 test isolates. These findings contradict the concept that EMB resistance is a direct consequence of multiple-drug resistance due to *embB*306 mutations that predispose isolates to facilitate broad drug resistance [6]. Instead, it is more likely that following the development of drug resistance to the powerful antituberculosis compounds (INH and rifampicin), subpopulations resistant to the less powerful EMB emerge due to increased selective pressure. Subtherapeutic plasma concentrations of INH in patients with a rapid acetylator status could create selective pressure that may lead to treatment failure and the emergence of drug resistance [25]. SNPs at *embB*306 displayed diverse MICs of which a significant proportion (73%) was scattered along the critical concentration resulting in borderline resistance. We conclude that borderline resistance can easily be missed by routine phenotypic drug susceptibility testing, hence false-susceptibility reporting. These data have clinical relevance and could be used to improve therapeutic strategies, especially in settings like the Western Cape (South Africa) where drug-resistant TB is problematic.

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Disclosure Statement

E.C. Böttger is a consultant for Becton Dickinson. All other authors have no conflict of interest to declare.

References

1 Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs WR Jr, Telenti A, Musser JM: Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of embB mutations. Antimicrob Agents Chemother 1997;41:1677–1681.

2 Starks AM, Gumusboga A, Plikaytis B, Shinnick TM, Posey JE: Mutations at *embB* codon 306 are an important molecular indicator of ethambutol resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2009;53:1061–1066.

3 Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchok B, Starks AM, Hooks DP, Cowan LS, Plikaytis BB, Posey JE: Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2011;55:2032–2041.

4 Plinke C, Walter K, Aly S, Ehlers S, Niemann S: *Mycobacterium tuberculosis embB* codon 306 mutations confer moderately increased resistance to ethambutol in vitro and in vivo. Antimicrob Agents Chemother 2011;55: 2891–2896.
5 Mokrousov I, Otten I, Vyshnevskiy B, Narvskaya O: Detection of embB mutations in ethambutol susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotype resistance testing. J Clin Microbiol 2002;40:3810–3813.
6 Hazbón MH, Bobadilla del Valle M, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, Colangeli R, Safi H, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, Davidow A, Brimacombe M, León CI, Porras T, Bose M, Chaves F, Eisenach KD, Sifuentes-Osornio J, Ponce de León A, Cave MD, Alland D: Role of *embB* codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. Antimicrob Agents Chemother 2005;49:3794–3802.
7 Böttger EC: Drug resistance in *Mycobacterium tuberculosis*: molecular mechanisms and laboratory susceptibility testing; in Donald PR, van Helden PD (eds): Progress in Respiratory Research: Antituberculosis Chemotherapy. Basel, Karger, 2011, vol 40, pp 128–144.
8 Madison B, Robinson-Dunn B, George I, Gross W, Lipman H, Metchock B, Sloutsky A, Washabaugh G, Mazurek G, Ridderhof J: Multicenter evaluation of ethambutol susceptibility testing of *Mycobacterium tuberculosis* by agar proportion and radiometric methods. J Clin Microbiol 2002;40:3976–3979.
9 Ahmad S, Jaber AA, Mokaddas E: Frequency of *embB* codon 306 mutations in ethambutol-susceptible and -resistant clinical *Mycobacterium tuberculosis* isolates in Kuwait. Tuberculosis 2007;87:123–129.
10 World Health Organization (WHO), Geneva, Switzerland: Policy guidance on drug-susceptibility testing (DST) of second line antituberculosis drugs. WHO/HTM/TB/2008.392:WHO, 2008, pp 1–20. http://www.who.int/tb/publications/2008/whohtm-tb_2008_392/en/index.html (accessed August 30, 2012).
11 Kremer K, van der Werf J, Au BKU, Anh DD, Kam KM, Rogier van Doorn H, Borgdorff MW, van Soolingen D: Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. Emerg Infect Dis 2009;15:335–339.
12 Streicher EM, Warren RM, Kewley C, Simpson J, Rastogi N, Sola C, van der Spuy GD, van Helden PD, Victor TC: Genotypic and phenotypic characterization of drug resistant *Mycobacterium tuberculosis* isolates from rural districts of the Western Cape Province of South Africa. J Clin Microbiol 2004;42:891–894.
13 Johnson R, Jordaan AM, Pretorius L, Engelke E, van der Spuy G, Kewley C, Bosman M, van Helden PD, Warren R, Victor TC: Ethambutol resistance by mutation detection. Int J Tuberc Lung Dis 2006;10:68–73.
14 Springer B, Lucke K, Calligaris-Maibach R, Ritter C, Böttger EC: Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* by use of MGIT960 and EpiCenter instrumentation. J Clin Microbiol 2009;47: 1773–1780.
15 Pasipanodya JG, Gumbo T: A new evolutionary and pharmacokinetic pharmacodynamic scenario for rapid emergence of resistance to single and multiple antituberculosis drugs. Curr Opin Pharmacol 2011;11:457–463.
16 Donald PR, Maher D, Maritz JS, Qazi S: Ethambutol dosage for the treatment of children: literature review and recommendations. Int J Tuberc Lung Dis 2006;10:1316–1330.
17 Safi H, Fleischmann RD, Peterson SN, Jones MB, Jarrahi B, Alland D: Allelic exchange and mutant selection demonstrate that common clinical *embCAB* gene mutations only modestly increase resistance to ethambutol in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2010;54:103–108.
18 Ramaswamy S, Musser JM: Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis 1998;79:3–29.
19 Goude R, Amin AG, Chatterjee D, Parish T: The arabinosyltransferase EmbC inhibited by ethambutol in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2009;53: 4138–4146.
20 Ramaswamy SV, Amin AG, Göksel S, Stager CE, Dou SJ, Sahly HEL, Moghazeh SL, Kreiswirth BN, Musser JM: Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2000;44:326–336.
21 Feuerriegel S, Oberhauser B, George AG, Dafae F, Richter E, Rüsch-Gerdes S, Niemann S: Sequence analysis for detection of first-line drug resistance in *Mycobacterium tuberculosis* strains from a high-incidence setting. BMC Microbiol 2012;12:90.
22 Plinke C, Cox HS, Zarkua N, Karimovich HA, Braker K, Diel R, Rüsch-Gerdes S, Feuerriegel S, Niemann S: *embCAB* sequence variation among ethambutol-resistant *Mycobacterium tuberculosis* isolates without *embB*306 mutation. J Antimicrob Chemother 2010;65:1359–1367.
23 Srivastava S, Musuka S, Sherman C, Meek C, Leff R, Gumbo T: Efflux-pump-derived multiple drug resistance to ethambutol monotherapy in *Mycobacterium tuberculosis* and the pharmacokinetics and pharmacodynamics of ethambutol. J Infect Dis 2010;201: 1225–1231.
24 Merza MA, Farnia P, Salih AM, Masjedi MR, Velayati AA: The most predominant spoligopatterns of *Mycobacterium tuberculosis* isolates among Iranian, Afghan-immigrant, Pakistani and Turkish tuberculosis patients: a comparative analysis. Chemotherapy 2010; 56:248–257.
25 Fahimi F, Kobarfard F, Tabarsi P, Hemmati S, Salamzadeh J, Baniasadi S: Isoniazid blood levels in patients with pulmonary tuberculosis at a tuberculosis referral center. Chemotherapy 2011;57:7–11.